REMARKS/ARGUMENTS

I. Status of the Claims

Claims 19-32 are pending.

II. The Present Amendment

No new matter has been added by the present amendments.

Claim 19 has been amended to clarify that it refers to full-length SEQ ID NO:2. Claim 20 has been amended to change the percent sequence identity. The change is supported throughout the specification, including page 6, lines 19-25, which refer to 85% sequence identity to a reference sequence. Claims 21 and 30 have been amended to recite that the peptide has a 90% sequence identity to SEQ ID NO:2 over a comparison window of about 10-20 amino acid residues. This language is supported throughout the specification, including page 6, lines 19-25. Claim 22 has been amended to correct a typographical error. Claim 23 has been amended to change the recitation of sequence identity as mentioned above in regard to claim 20, and to add the functional recitation present in claims 21 and 30. Claim 27 has been amended to clarify that the peptide is isolated, and to correct a typographical error. Claim 32 has been amended to correct its antecedence.

III. The Office Action

The Action rejects the claims on a number of grounds. Applicants amend in part and traverse all the rejections. For the reader's convenience, the rejections posed by the Action are considered below in the order in which they appear in the Action.

A. Rejections of Claims 19-32 Under 35 U.S.C. § 112, first paragraph as not enabled

The Action rejects claims 19-32 under 35 U.S.C. § 112, first paragraph, as allegedly not enabled. According to the Action, the specification teaches that SEQ ID NO:2

appears to be a differentiation antigen expressed on mesothelioma and some ovarian cancer cells, but also teaches that SEQ ID NO:2 is very abundant on normal mesothelial cells. The Action asserts that the:

> "specification does not teach that SEQ ID NO:2 is differentially expressed in normal mesothelial cells compared with malignant mesotheliomas and ovarian cystadenocarcinomas. . . . If indeed there is no differential between the malignant tissue expression and the normal expression, it is not clear how the expression of SEQ ID NO:2 could be used to detect cancer and it is certainly unclear as to how to use SEQ ID NO:2 to treat cancer, given that the specification specifically teaches that the use of the antigen in treatment would be expected to damage normal mesothelial cells as well as cells of the trachea. The specification provides insufficient guidance with regard to these issues and provides no working examples . . . For the above reasons, it appears that undue experimentation would be required to practice the invention."

Action, at pages 4-5, bridging paragraph. Applicants traverse.

As set forth above, the rejection is based entirely on the alleged lack of enablement for the use of the isolated protein and peptides of the invention as vaccines. Applicants respectfully note, however, that the claims do not recite vaccines, but isolated proteins and peptides. The claimed proteins and peptides are useful, for example, to raise antibodies, as stated in the specification at page 14, lines 28-31. In turn, these antibodies are useful, for example, for detecting the presence of mesothelin in a biological sample, as discussed at page 26, lines 29-38, and for targeting cytotoxins to cells expressing mesothelin, as discussed at page 28, lines 5-18. The Action neither alleges nor shows that raising antibodies by the claimed proteins and peptides is not an appropriate utility or is not enabled.

The Examiner is respectfully reminded that a composition requires only one utility to be patentable, and that it only need be enabled for that utility. The fact that the composition may also be useful for other purposes is therefore irrelevant. Applicants respectfully request reconsideration and withdrawal of the rejection.

B. Rejection of claims 19-25 and 27-32 Under 35 U.S.C. § 112, first paragraph as not enabled

The Action further rejects claims 19-25 and 27-32 under §112, first paragraph, as not enabled for an isolated protein comprising a full-length amino acid sequence of SEQ ID NO:2, isolated proteins having 90% sequence identity to SEQ ID NO:2 which meets certain functional recitations, and isolated peptides of at least 10 contiguous amino acids of SEQ ID NO:2 which meet certain functional recitations. The rejection sets forth several distinct arguments as to why this is assertedly true. Applicants amend in part and traverse. Because of the length of the rejection, which extends over some 13 pages, the Action's contentions, and Applicants' responses to those contentions, are set forth separately below for the reader's convenience.

1. Allegations that variants of the protein and peptides may not exist on cells

According to the Action:

> "[o]ne cannot extrapolate the teaching of the specification to the scope of the claims because the specification provides no guidance on whether any variant, as claimed, is expressed on any cell and given the novelty of the claimed invention, it cannot be predicted, based on the information in the specification or the art of record whether such a variant exists."

Action, at page 8. Applicants traverse.

The rejection is a *non sequitor*. The claims as presented are drawn to the mesothelin sequence, SEQ ID NO:2, to proteins that have a defined degree of sequence identity to SEQ ID NO:2 and which retain the ability to raise antibodies which bind to the protein of SEQ ID NO:2, to peptides of contiguous amino acids of SEQ ID NO:2 (that is, to fragments of the mesothelin protein) which have this functionality, and to peptides which have a defined degree of sequence identity to the mesothelin fragments and which retain the ability to raise antibodies which bind to the protein of SEQ ID NO:2. There is no requirement in the recitation of the

claims that the "variants" of the protein or of the mesothelin fragments be expressed on a cell, nor is that necessary for the claimed "variants" to have utility or to be used by practitioners.

It is respectfully noted that the recitation of proteins and peptides which have a defined degree of sequence identity to a particular novel protein, and which retain a particular function, is a common claim format. The proteins and peptides of the claims presented have the function of being able, for example, of raising antibodies which bind to the protein of SEQ ID NO:2. Accordingly, the rejection rejects the claims on an inapplicable basis.

2. Allegations that protein chemistry is unpredictable

According to the Action:

> even if a function of SEQ ID NO:2 were known at the time the invention was made, it is well known in the art that the art of protein chemistry is highly unpredictable.

Action, at page 9. The Action then cites a series of references, Bowie et al., Science, 257:1306-1310 (1990); Burgess et al., J Cell Biol 111:2129-2138 (1990); and Lazar, Mol Cell Biol, 8:1247-1252 (1988), for the proposition that protein chemistry is unpredictable, in that the substitution of a single amino acid can destroy protein function, and cites Scott, Nat Genetics 21:440-443 (1999) and Bork, Genome Res, 10:398-400 (2000), for the proposition that sequence similarity data and computational sequence analysis is far from perfect. According to the Action:

> given not only the teachings of Bowie et al, Lazar et al, Scott et al and Burgess et al but also the limitations and pitfalls of using computational sequence analysis and the unknown effects of alternative splicing, post translational modification and cellular context on protein function as taught by Bork, with a 10% dissimilarity to SEQ ID NO:2 , the function of the claimed variants, even if that of SEQ ID NO:2 were known, could not be predicted, based on sequence similarity with SEQ ID NO:2, nor would it be expected to be the same as that of SEQ ID NO:2.

Action, at pages 9-12. Applicants traverse.

Applicants respectfully note that this rejection might have some merit if the claims under rejection recited a protein with, for example, an enzymatic or therapeutic activity, but has little apparent application to the claims actually presented. The claims under examination are not drawn to proteins or peptides that have a delicate or special activity that might be unpredictably destroyed by a single mutation; they need only be capable of, for example, raising antibodies to the protein of SEQ ID NO:2. Proteins and peptides that do not meet one of the functions recited by the claims are not within their scope.

The Examiner is respectfully reminded that claims can encompass non-working embodiments, so long as there is a test by which the person of skill can readily determine whether or not a composition is or is not within the scope of the claims. As set forth by MPEP §2164.08(b), the "standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is normally required in the art. *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1577, 224 USPQ 409, 414 (Fed. Cir. 1984) (prophetic examples do not make the disclosure nonenabling)."

The claims under examination meet the MPEP's standard. The person of skill can readily determine whether any particular protein or peptide can, for example, raise antibodies that bind to the protein of SEQ ID NO:2 by injecting the protein or peptide into a mouse or other suitable animal, as described for example in the specification at page 15, line 15, to page 16, line 16, and determine whether any antibodies raised by the mouse recognize the protein of SEQ ID NO:2 using immunoassays standard in the art, such as ELISAs. Applicants respectfully observe that injecting animals with peptides and determining whether or not antibodies are raised is a common practice in the art (as well as being described in the specification) and is, in the words of MPEP §2164.08(b), "an expenditure of no more effort than is normally required in the art."

The Bowie, Burgess, Lazar, Scott and Bork references are not relevant to the claims under examination. First, the references are not analogous to the present invention. The references relate to the biological activity of proteins; none of the references relate to the

production of antibodies to a protein, and therefore lend no support to the Action's contentions that a change of an amino acid could affect the ability of the protein or peptide to raise antibodies recognizing SEQ ID NO:2. Second, as shown below, the specific teachings of these references either support the claims under examination, or are not relevant to them.

Bowie is the most general of the references. The Action cites Bowie for the proposition that the change of a single amino acid in the sequence of a protein can destroy its function. Even assuming this proposition has any relevance to the claims under examination, which, as noted above, is not correct, the Action's contention takes Bowie's statement out of context and thus fails to consider Bowie's teachings as a whole. Bowie's teaching as a whole is "that proteins are surprisingly tolerant of amino acid substitutions . . . For example, . . . about one-half of all substitutions [made in a study substituting every position in the *lac* repressor] were phenotypically silent [citation omitted] At some positions, many different, nonconservative substitutions were allowed." Bowie, at page 1306, right column, first full paragraph (emphasis added). Thus, even in terms of maintaining a protein's biological activity, Bowie teaches that about one half of the residues can be changed without effect and that many nonconservative substitutions can be made. In light of Bowie, persons of skill would predict that many substitutions can likely be made in SEQ ID NO:2 or in fragments of SEQ ID NO:2 without affecting the ability to generate antibodies that bind the protein of SEQ ID NO:2 or of T-cells activated by endogenous mesothelin in a patient with a mesothelin-expressing cancer to recognize the protein or fragment.

The Lazar reference relates to the mutation of two specific amino acids known to be conserved in the same family of peptides as the peptide, TGF-α, that is the subject of the reference. The particular mutations made were calculated to change the characteristics of the amino acid at the conserved position. For example, the authors mutated an aspartic acid (which would normally be negatively charged at physiological pH) at position 47 of the molecule to an alanine or an asparagine (which would normally be uncharged at physiological pH). See, abstract. Not surprisingly, perhaps, these non-conservative mutations of a conserved residue

changed the biological activity. There is no evidence or teaching in the reference, however, that even this mutated protein would not have raised antibodies that bind to the original TGF-α.

Burgess concerns the mutation of a residue known to be important to the biological activity of a particular growth factor (HBGF-1). Like Lazar, the mutation made is a non-conservative mutation (from positively charged lysine to negatively charged glutamic acid), specifically because the lysine in question had been previously implicated as important to the biological activities of the growth factor. See, abstract. Not surprisingly, this deliberately non-conservative change in a key residue affected activity. What is not clear is what relevance this finding has to the generation of antibodies to a protein, such as the growth factor, or of activated T cells to recognize it. If anything, the reference would appear to support the present claims, since even this non-conservative mutation did not affect the binding of the growth factor to its receptor (see, Burgess at page 2135, right column, last paragraph above figure: "The receptor-binding properties of the mutant HBGF-1 are not distinguishable from those of the wild-type protein . . . "). Thus, to the extent the reference has any relevance to binding of a ligand to its binding partner, the reference shows that binding was not affected by the mutation.

Scott is intended by the Action to support the contention that it is difficult to predict protein function even of a protein with close homology to other proteins with a known function. In this regard, the reference reports that the closely homologous proteins transported sulfate ions, while the product of the Pendred syndrome gene turned out not to transport sulfate ions, but rather chloride and iodide ions. This reference might therefore be relevant if the claims were drawn to proteins having a biological function similar to that of native mesothelin. But, they do not: they are drawn merely to proteins and peptides that can raise antibodies that recognize mesothelin or that are recognized by T cells activated to recognize mesothelin. Scott simply provides no evidence that variants of the protein of SEQ ID NO:2, or fragments thereof, would be incapable of this function. Further, since the reference does not concern mutating a sequence, it appears to be even less relevant than Lazar and Burgess to the claims under examination. And even were the point for which the reference is cited relevant to the present claims, which Applicants respectfully maintain it is not, Applicants note that while the anion

transported may have been different, the gene product of the Pendred syndrome gene was still an anion transporter. Therefore, the homology to other transporters still predicted the overall function of the molecule.

Finally, the Action raises Bork presumably to support its contention that one must consider "computational sequence analysis and the unknown effects of alternative splicing, post translational modification and cellular context on protein function." These concerns seem particularly poorly applied to the claims as presented. Once again, Bork might have been relevant if the claims were drawn to predicting proteins having a biological function similar to that of native mesothelin. But, they are not: they are drawn merely to proteins and peptides that can raise antibodies that recognize mesothelin or that are recognized by T cells activated to recognize mesothelin. Bork provides no indication that this is either unpredictable or difficult.

The Action's concerns regarding "alternative splicing, post translational modification and cellular context on protein function" are hypotheticals - the Action presents no evidence that any of these factors are present with regard to SEQ ID NO:2. SEQ ID NO:2, however, is not a hypothetical protein, but one isolated as described in the specification and expressed particularly on cells of certain cancers. Even assuming that some alternative splicing of the gene that encodes SEQ ID NO:2 occurs in nature (which, as noted above, the Action does not show), it would be irrelevant to the patentability of the proteins and peptides of the claims under examination. Applicants are aware of no requirement in the patent law that the claimed proteins and peptides be able to raise antibodies that bind not only to SEQ ID NO:2 but also to all possible alternative forms; to the contrary, the utility and enablement requirements are satisfied so long as the claimed proteins and peptides raise at least some antibodies that bind to one form of the protein and those antibodies have a non-frivolous use. The proteins and peptides claimed herein are useful precisely because they raise antibodies that bind to the protein of SEQ ID NO:2. Thus, even if alternative splicing of the gene that encodes SEQ ID NO:2 were shown to exist - and the Action presents no evidence that it does - it would be irrelevant to the examination of the claims.

Similarly, Applicants respectfully maintain that the Action's speculation about possible post-translational modifications in the expression of SEQ ID NO:2 is irrelevant to the claims as presented. There is no requirement that the antibodies bind to every form of mesothelin that might exist.

Finally, the Action's statement regarding the "cellular context on protein function" is presumably drawn from Bork's comments regarding "higher order functions that involve concentration, compartmental organization, dynamics" etc. These assertions again might be relevant if the claims were drawn to predicting proteins having a biological function similar to that of native mesothelin. But, they are not: they are drawn merely to proteins and peptides that can raise antibodies that recognize mesothelin or that are recognized by T cells activated to recognize mesothelin. Concerns about "higher order functions" such as "compartment organization" appear especially irrelevant to an antigen presented on the cell surface, rather than in a cytosolic organelle. In short, these concerns are simply not applicable to the claims as presented.

Applicants respectfully request reconsideration and withdrawal of this aspect of the rejection.

3. Allegations that specification does not teach specific epitopes

The Action next raises a series of arguments to the effect that the proteins and peptides as claimed are not enabled because there is allegedly some unpredictability as to the epitopes that are immunogenic. Applicants traverse all of the arguments.

i.) Argument that it cannot be determined whether the antibodies actually bind SEQ ID NO:2

According to the Action:

> As drawn specifically to the claimed isolated variant peptides used as immunogens, as suggested by the specification, for raising antibodies that recognize full-length SEQ ID NO:2, even if the peptides claimed were 100%

> identical to portions of SEQ ID NO:2 it would not be possible to determine with any predictability whether the antibodies produced from a fragment that is specific for SEQ ID NO:2 actually bind to SEQ ID NO:2."

Action, at page 12.

The Action's statement that "it would not be possible to determine with any predictability whether the antibodies produced from a fragment that is specific for SEQ ID NO:2 actually bind to SEQ ID NO:2" is puzzling. It is not clear what the Action means by "a fragment that is specific for SEQ ID NO:2." It appears to relate to actual fragments of SEQ ID NO:2, as opposed to peptides that have 90% sequence identity to a fragment of SEQ ID NO:2. But, either way, the contention is without merit. Whether or not antibodies raised by any particular protein or peptide actually bind to SEQ ID NO:2 can be easily accomplished by standard immunoassays, such as ELISAs. If the Examiner chooses to maintain this portion of the rejection, Applicants respectfully request that the Examiner explain why these standard techniques in the art, in use for decades, would not be successful in determining whether the antibodies raised would "actually bind to SEQ ID NO:2." In the absence of a credible explanation, Applicants respectfully maintain that this portion of the Action's argument should be reconsidered and withdrawn.

ii.) Argument that 3-dimensional structure of SEQ ID NO:2 cannot be predicted

The Action next asserts that:

> it cannot be predicted, given the information in the specification, which of the sequences are exposed on the surface of SEQ ID NO:2. . . . [the regions that are immunogenic] are often at exposed areas on the outside of the antigen. Furthermore, the specification does not take into account the 3 dimensional folding of the native molecule, nor its glycosylation or other post-translational modifications and other characteristics . . . Peptides or synthetic antigens cannot effectively substitute for the natural tertiary and quaternary structure in a physiological situation. Further there is no teaching in the specification of which

> part of the protein should be used to produce antibodies which will bind specifically to SEQ ID NO:2. . . . as written, the claims encompass defined specific epitopes of SEQ ID NO:2. However, there is no teaching in the specification of whether or not the epitopes are linear or comprise 3-dimensional structures. . . . Antibodies bind to structural shapes that may be linear stretches of amino acids, conformational determinants formed by the folding of peptides, carbohydrate moieties, phosphate or lipid residues or a combination thereof.

Action, at pages 12-13.

As an initial matter, Applicants respectfully remind the Examiner that the tertiary structure of a molecule is determined by its primary structure (i.e., the sequence of its amino acids). Therefore, the tertiary structure of SEQ ID NO:2 is necessarily determined by its amino acid sequence (which of course is set forth as SEQ ID NO:2). It is possible that proteins with 90% sequence identity to SEQ ID NO:2 will have a different conformation, and therefore different conformational epitopes. It is unlikely, however, that they will not have portions that do not have linear or conformational epitopes that will raise antibodies that also bind to SEQ ID NO:2 and, of course, if they do not, they are not within the scope of the claims as presented. The Examiner is respectfully reminded that MPEP § 2164.04(b) permits the claims to encompass inoperable embodiments so long as the practitioner can determine whether or not the embodiment is operable with no more effort than is normally used in the art. The practitioner can test any particular protein by tests such as those noted in the preceding section. Such tests are commonly conducted in the art, and accordingly meet the standard articulated by § 2164.04(b).

With respect to peptides that are fragments of SEQ ID NO:2 or that share 90% identity with such fragments, such peptides may well only relate to linear epitopes, not to conformational epitopes that might be created by the overall 3-dimensional structure of the protein of SEQ ID NO:2. Antibodies against linear epitopes are useful, for example, to bind to denatured protein of SEQ ID NO:2 in SDS-PAGE and Western blots. Applicants are aware of no requirement of the patent law that the claimed peptides have to also raise antibodies against

conformational epitopes. Further, as pointed out in the definition of "epitope," in the excerpt from the Herbert et al. "Dictionary of Immunology" (4th Ed., 1995) provided with the Action, epitopes recognized by T cells are peptide fragments of a continuous primary sequence presented in conjunction with an MHC class I molecule. Thus, persons of skill were aware as of the time of filing that while antibodies may be raised by linear or by conformational epitopes, T cells recognize linear epitopes.

The Action's comments about glycosylation are not well taken. As shown in Figure 2, there are only 4 glycosylation points on SEQ ID NO:2. The Action presents no evidence or reference to show that the presence of a few carbohydrates would prevent the generation of antibodies to other portions of the protein. Moreover, even if the presence of these carbohydrates prevented the generation of any antibodies to the glycosylated protein, which the Action neither alleges nor shows, it would not affect the utility of antibodies to SEQ ID NO:2 to identify the protein in *in vitro* assays following use of deglycosylating enzymes well known in the art (see, e.g., U.S. Patent No. 5,238,821) to remove the carbohydrate groups. While the Action speculates about the effect of other post-translational modifications, none have been shown or suggested.

Applicants respectfully maintain that this portion of the Action's argument should be reconsidered and withdrawn.

iii.) Arguments that it is not predictable that variants of peptides would raise antibodies that bind SEQ ID NO:2

The Action next asserts:

> as evidenced by Greenspan et al., defining epitopes is not as easy as it seems (Nature Biotechnology 7:936-937 (1999). . . . Since the specification has not identified which amino acids and or polypeptide fragments are critical or essential characteristics of the epitope, it would not be predictable that the claimed peptides would in fact be specific epitopes of SEQ ID NO:2 or that antibodies produced against these peptides would in fact bind to SEQ ID NO:2. Given the

> above, given the teachings of Bowie et al that an amino acid sequence encodes a message that determines the shape and function of a protein and that it is the ability of these proteins to fold into unique three-dimensional structures that allows them to function and teachings of Burgess et al, Lazar et al, Scott et al drawn to the effects of the alteration of even a single amino acid, even if the specific peptides of the invention were found to produce antibodies that bind to SEQ ID NO:2, it could not be predicted that the claimed variants would also function to produce antibodies that would bind to SEQ ID NO:2.

Action, at pages 13-14.

The argument that the specification has not defined specific epitopes is an argument that the peptides will not raise antibodies that will bind SEQ ID NO:2. The Examiner is respectfully reminded that MPEP § 2164.04(b) permits the claims to encompass inoperable embodiments so long as the practitioner can determine whether or not the embodiment is operable with no more effort than is normally used in the art. The practitioner can test any particular protein by tests such as those noted in the preceding section. Such tests are commonly conducted in the art, and accordingly meet the standard articulated by § 2164.04(b). It should further be noted that an invention can require considerable experimentation to practice so long as that experimentation is not "undue." While the number of peptides that can be generated meeting the claim criteria may be extensive, each of them can be tested by routine procedures practiced within the art since at least the 1960's. Accordingly, Applicants maintain any experimentation necessary may be "considerable", but is not "undue."

The attempt to apply Bowie, Burgess, Lazar and Scott to suggest that the change in a single amino acid might destroy the ability of the claimed peptides to raise antibodies that bind SEQ ID NO:2 is not appropriate. As noted above in connection with the citation of these references, the references do not regard matters analogous to the present invention. These references relate to the biological activity of the protein; none of the references relate to the production of antibodies to a protein, and therefore lend no support to the Action's contentions.

Second, as shown below, the specific teachings of these references either support the claims under examination, or are not relevant to them.

As noted above, Bowie is the most general of the references. As quoted above, the Action cites Bowie for the proposition that the amino acid sequence of a protein contains all the information necessary to determine its structure. Bowie, at page 1306, left column. But, the Action fails to note that Bowie shortly thereafter also states "that proteins are surprisingly tolerant of amino acid substitutions . . . For example, . . . about one-half of all substitutions were phenotypically silent [citation omitted] At some positions, many different, nonconservative substitutions were allowed." Bowie, at page 1306, right column, first full paragraph. Thus, assuming Bowie is applicable at all to analysis of the claims under examination, it would lead persons of skill to expect that many substitutions - and certainly 10% or fewer - could be made in fragments of SEQ ID NO:2 without affecting the ability to generate antibodies that bind the protein of SEQ ID NO:2 or to T-cells activated by endogenous mesothelin in a patient with a mesothelin-expressing cancer.

The Lazar reference relates to the mutation of two specific amino acids known to be conserved in the same family of peptides as the peptide, TGF-α, that is the subject of the reference. The particular mutations made were calculated to change the characteristics of the amino acid at the conserved position, such as mutating an aspartic acid (which would normally be negatively charged at physiological pH) at position 47 of the molecule to an alanine or an asparagine (which would normally be uncharged at physiological pH). See, abstract. Not surprisingly, perhaps, these non-conservative mutations of a conserved residue changed the biological activity. There is no apparent evidence in the reference, however, that this mutated protein would not have raised antibodies that bind to the original TGF-α.

Burgess concerns the mutation of a residue known to be important to the biological activity of a particular growth factor (HBGF-1). Like Lazar, the mutation made is a non-conservative mutation (from positively charged lysine to negatively charged glutamic acid), specifically because the lysine in question had been previously implicated as important to the biological activities of the growth factor. See, abstract. Not surprisingly, the activity was

affected. What is not clear is what relevance this finding has to the generation of antibodies to a protein, such as the growth factor. If anything, the reference would appear to support the present claims, since even this non-conservative mutation did not affect the binding of the growth factor to its receptor (see, Burgess at page 2135, right column, last paragraph above figure: "The receptor-binding properties of the mutant HBGF-1 are not distinguishable from those of the wild-type protein . . . "), which suggests that to the extent the reference has any relevance to binding of a ligand to its binding partner, the binding was not affected by the mutation.

Scott adds nothing to the Action's contention, since it merely concerns the ability to predict the ion transported by the product of the Pendred syndrome gene by its homology to other transporters. (Applicants note in passing, however, that while the substrate may have been unpredictable, the transporter did still act as an ion transporter, as its homology suggested.) Since it does not concern mutating a sequence, it appears to be even less relevant than Lazar and Burgess to the claims under examination.

In short, the references cited by the Action do not support the thesis of the rejection. The rejection should be reconsidered, and withdrawn.

iv.) Arguments re unpredictability of which peptides would comprise T cell epitopes

The Action next asserts:

> it would not be possible to determine with any predictability which of the peptides of SEQ ID NO:2 would be recognized by T cells from cancer patients. In particular, Kirkin et al, 1998, APMIS, 106:665-679 et al [sic] teach that in particular for tumor antigens, for some antigens, due to the existence of self-tolerance, only T cells with low affinity T-cell receptors are produced (abstract). Further, Chaux et al, Int J Cancer, 1998, 77:538-542 teach some of the CTLs have an affinity that is too low for the recognition of cells that have processed the antigen, which is different from the in vitro conditions in which the synthetic peptides are in high number when incubated with the cells . . . Given the above,

> even if a peptide was recognized by T-cells in vitro from patients with [mesotheliomas] or ovarian cancer cells expressing mesothelin, it could not be predicted that the T-cells would recognize these peptides *in vivo* and if not recognized *in vivo*, it is clear one would not know how to use the claimed peptides. Similarly, Sherman LA et al 1998, Critical reviews in Immunol, 18(1-2):47-54 teach that self-tolerance may eliminate T cells that are capable of recognizing T-cell epitopes with high avidity. Smith RT, 1994, Clin Immunol, 41(4):841-849, teaches that antigen overload, due to antigen shedding by actively growing tumor, could block specifically either cytotoxic or proliferative responses of tumor specific T cells.

Action, at pages 14-15.

As an initial matter, Applicants note that there is no logical nexus between the Action's statement that it would not be possible to determine which peptides of SEQ ID NO:2 would be recognized by T cells from cancer patients, on the one hand, and the alleged teachings, of the Kirkin and Chaux references on the other. Even if the Kirkin and Chaux references were relevant to the invention as claimed (as shown below, they are not), it would not be clear that alleged teachings of Kirkin that for some tumor antigens only low affinity T cell receptors are produced and of Chaux that some CTLs have too low affinity for the recognition of antigen provide any support for the Action's proposition that there would be difficulty in identifying peptides that would be recognized by T cells from cancer patients. Applicants respectfully note that producing T cells with too low an affinity (even if true) is not the same thing not producing T cells that recognize the antigen.

In any event, it is respectfully noted that Kirkin and Chaux are not relevant to the claims under examination. Kirkin is concerned exclusively with melanoma-associated antigens. The Action does not explain why this review of findings with regard to melanoma antigens has any bearing on the immunogenicity of mesothelin, which is not a melanoma antigen. It is not appropriate for the Action to simply assume the underlying premise that findings for melanoma antigens are common to other cancer antigens or to cancer antigens in general. Nothing in the

reference states this or indicates it is true. In the absence of a showing of some nexus, there is no basis on which the Action can argue that Kirkin's teachings are applicable to the claims presented.

Likewise, Chaux does not support the Action's case. The Chaux reference concerns the development of an extremely sensitive immunoassay for detecting CTL precursors. Since the authors detected more peptide-HLA class complexes following incubation with a synthetic peptide than they did following endogenous processing, they speculated that some of the CTLs that scored positive had an affinity that was too low for the recognition of cells that processed the antigen endogenously. Chaux, at page 541, right column, first full paragraph. Applicants again observe, however, that producing T cells with too low an affinity is not the same thing not producing T cells that recognize the antigen.

Further, the authors' speculation regarding affinity is an attempt to explain why they counted more complexes in connection with the peptide than they did in connection with endogenously processed antigen. But, while the authors state: "we could not exclude that some of the CTLs that scored positive in our assay have an affinity that is too low for the recognition of cells that have processed the antigen." Chaux, at page 541, right column (emphasis added). They evidently believed, therefore, that the majority of the T cells produced by incubation with the peptide had sufficient affinity for the recognition of cells that had processed the antigen. Thus, Chaux does not provide a teaching that antigen peptides cannot produce CTLs that have sufficient affinity to bind to cells that have processed the antigen. Applicants also respectfully note that, if a particular variant of SEQ ID NO:2 or of a fragment of SEQ ID NO:2 is not recognized by T cells from patients with mesothelin-expressing cancers, it is not within the scope of the claims.

While the two references do not stand for the proposition for which they are presented, for extra measure, Applicants also take this opportunity to note that, in 1998, the year both of these references were published, persons of skill were using adjuvants to increase the robustness of the immune response to cancer antigens. For example, Jager et al., Recent Results Cancer Res 152:94-102 (1998) indicates that while tumor-associated antigens alone elicit

delayed type hypersensitivity (DTH) and CTL responses leading to tumor regression after intradermal injection, GM-CSF was effective at enhancing such reactions. Similarly, Dillman et al., Cancer Biother Radiopharm 13(3):165-76 (1998) indicate that patients who received interferon gamma or GM-CSF in addition to autologous metastatic melanoma tumor cells showed a higher rate of DTH conversion compared to patients administered other adjuvants. (For the Examiner's convenience, copies of the abstracts of Jager et al. and Dillman et al. are enclosed.) Thus, even assuming that Kirkin and Chaux taught in 1998 that peptide antigens do not by themselves raise enough of an immune response from CTLs to be used in vivo, the art was aware at the same time that adjuvants could be used to augment that response. Applicants note that the specification sets forth a considerable list of exemplar adjuvants that can be used in conjunction with the claimed peptides, as well as carrier molecules that can themselves be expected to augment immune response. See, specification at pages 44-45, bridging paragraph, and page 45, lines 15-30.

The Action's comment that if the peptides of the invention cannot be used *in vivo*, it is not clear how to use the claimed peptides is without merit. It was known in the art at the time of filing that dendritic cells could be pulsed or loaded with tumor antigen peptides *in vitro* and that such dendritic cells could be used to develop specific T-cell responses *in vivo*. See, e.g., Mayordomo et al., Nat Med 1(12): 1297-1302 (1995), Paglia et al., J Exp Med 183(1):7-11 (1996), Porgador et al., J Immunol 156(8):2918-26 (1996) (copies of the abstracts of these references are enclosed for the Examiner's convenience). Thus, even assuming that the Action's contentions regarding *in vivo* use were correct, which Applicants do not concede, the practitioner would still be enabled to use the invention *ex vivo*.

Sherman's concerns that self-tolerance may eliminate T cells capable of recognizing T cell epitopes with high avidity is answered by the fact that Sherman itself indicates that animals primed with the antigen can eliminate tumors with the self antigen. Given that pulsing or loading dendritic cells with antigen to prime T cells was known at the time of the invention, as shown above, Applicants maintain that techniques already existed to break self-tolerance adequately to enable the invention as claimed.

As noted, Smith is cited for the proposition that antigen overload due to antigen overload could block development of T cell responses. Smith itself notes, , however, that this anergy can be overcome in vitro by use of cytokines. Perhaps it was the findings noted in Smith regarding the use of cytokines that led the better results in the studies mentioned above in which cytokines such as GM-CSF used in conjunction with tumor antigens increased DTH and CTL response. Finally, the Action cites page 484 of Smith as indicating that tumors progressively lose MHC representation at the surface of the cell. This page does not appear in the reference of Smith provided with the Action and Applicant could not locate a section making this asserted point. The Examiner is requested to clarify the reference to the publication if this portion of the rejection is maintained. Applicants respectfully note, however, that the present claims are drawn to compositions, not methods. Moreover, even if tumors lose MHC representation over time, that does diminish the value of raising a T cell response early in treatment of a cancer. Nothing in the patent statute requires that a composition be useful throughout the entire course of a disease. It need be useful only for a time to have utility.

Reconsideration, and withdrawal, of this portion of the rejection is respectfully requested.

v.) Arguments that peptides read on a vaccine

The Action asserts:

although Applicant has amended the claims to delete reference to a vaccine . . . the claims . . . still read on a vaccine since the only contemplated use for peptides as drawn to T- cell's is for vaccination . . . Thus, essentially for the reasons previously set forth in the Paper mailed December 19, 2001, Section 6, pages 3-5, claims 2-32 are rejected. . . . Further, as drawn to cancer vaccines, Boon (Adv Can Res, 1992, 58:177-210) teaches that for active immunization in human patients we have to stimulate immune defenses of organisms that have often carried a large tumor burden. Establishment of immune tolerance may therefore have occurred . . . the therapeutic success remains unpredictable due to inconsistencies in antigen expression or presentation by tumor cells . . .Thus based on the teaching in the art and in the specification one cannot

predict that an adequate in vivo T cell response useful for immunotherapy, as contemplated, could be induced by the peptides of the invention in having [sic] tumor burden. In addition . . . Kirkin et al. . . . conclude that initiation of a strong immune response in vivo is an extremely rare event . . . for some antigens, due to the existence of self-tolerance, only T cells with low affinity T cell receptors are produced.
Action, at page 15-17.

As an initial matter, Applicants note that this aspect of the rejection is premised on the Action's conclusion that the only contemplated use for the peptides of the invention, as drawn to recognition by T cells, is as a vaccine. This is not correct. As pointed out above, it was known in the art at the time of filing that dendritic cells could be pulsed or loaded with tumor antigen peptides *in vitro* and that such dendritic cells could be used to develop specific T-cell responses *in vivo*. See, e.g., Mayordomo et al., Nat Med 1(12): 1297-1302 (1995), Paglia et al., J Exp Med 183(1):7-11 (1996), Porgador et al., J Immunol 156(8):2918-26 (1996). Thus, persons of skill in the art at the time of filing would be immediately aware that they could use peptides from mesothelin, the tumor antigen disclosed in the present specification, to pulse or load dendritic cells *in vitro*.[1] Further, it was also known in the art at the time of the invention that dendritic cells could be transfected with a gene encoding a tumor antigen, and that the transfected dendritic cells could process and present the antigen. See, e.g., Alijagic et al., Eur J Immunol. 25:(11):3100-7 (1995); Bakker et al. Cancer Res. 55(22):5330-4 (1995). (copies of the abstracts of Alijagic et al. and Bakker et al. are enclosed for the Examiner's convenience). Thus, it was known that dendritic cells could be transfected, for example, with a gene encoding variants of SEQ ID NO:2, which the cells would then process and express on their surfaces. Therefore, contrary to the Action's assertion, the were and are *in vitro* uses for the peptides of the invention.

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[1] It is irrelevant that this use may not have been set forth in the specification in so many words. The well established rule is that "the specification not need teach, and preferably omits, that which is well known in the art." *See, e.g., In re Buchner*, 18 USPQ2nd 1331, 1332 (Fed. Cir. 1991). Accordingly, if a use assay for a claimed peptide would be immediately apparent to one of ordinary skill in the art, it does not need to be set forth expressly in the application.

Next, the Action states that the claims are rejected for "essentially the reasons" set forth at pages 3-5 of the Office Action of December 2001. The Action fails to note, however, that the contentions set forth in those pages were carefully answered and shown to be deficient in the Applicants' June 19, 2002 Amendment. Proper examination practice requires that, if the Examiner chooses to maintain a rejection in the face of an applicant's response, that the applicant's response be carefully considered and an explanation given why the response is not sufficient to overcome the rejection. The Action's simple reiteration of the previous rejection does not even acknowledge the Applicants' June 2002 response, let alone address its contentions. The rejection should be reconsidered and withdrawn on this basis alone.

The Action's reliance on Boon as showing that there may be problems with immune tolerance is not well placed. Even assuming that Boon's speculation about tolerance was correct (and it should be noted that his remarks indicate only that tolerance "may therefore have occurred") and that patients with more advanced disease and a "large tumor burden" might have a degree of tolerance of the tumor, that does not indicate that patients with less advanced disease and a lower tumor burden would have the same degree of tolerance. But, Boon does not indicate what would happen if the tumor rejection antigens were administered to a patient with a less advanced cancer and, consequently, a low tumor burden. Boon provides no support for the proposition such an individual would have immune tolerance or depressed capability to respond. Accordingly, Boon does not indicate that there is no population of patients of mesothelin-expressing cancers that would have an increased immune response to the cancer as the result of administration of the peptides of the invention. It is respectfully noted that the claims do not recite that the proteins and peptides are only for administration to persons with large tumor burdens; there is no evident reason they could not be given to individuals with early stage disease and as-yet modest tumor burden.

The Action indicates that Boon states at page 178 that there is inconsistent antigen expression and presentation by tumor cells even if activated CTLs are significantly increased. Action, at page 16. Applicants could not locate the passage of Boon the Action asserts makes this point, and therefore could not fully evaluate its context. They note, however, there is no

requirement that the proteins and peptides of the invention induce an immune response in every patient. Further, to the extent that the Boon passage suggests that a cancer antigen may not be expressed on all cells of a cancer and therefore might not be therapeutically useful, Applicants note that it is useful to the practitioner and to the patient if disease progression is reduced by affording some response to those cells of the tumor that do express the antigen. Accordingly, even if the Action were correct that it is unpredictable that all cells in a mesothelin-expressing cancer tumor express mesothelin, which Applicants do not concede, it would be irrelevant to the utility and to the enablement of the claims under examination.

The Action next contends that Kirkin shows that initiation of a strong immune response in vivo is a very rare event. The Action notes that, of the MAGE-3 peptides discussed by Kirkin, only one had limited anti-tumor activity. With respect, the Action's contention might be applicable if the claims were drawn to peptides with anti-cancer effects in general. But, they are not: they are drawn specifically to interactions with T cells from patients with mesothelin-expressing cancer. In contrast, Kirkin is specifically directed to melanoma-associated antigens. The Action does not show (and does not even attempt to show) that the Kirkin discussion with respect to melanoma-associated antigens is relevant to cancers expressing mesothelin, which melanoma cells do not. Applicants respectfully note that it is the Examiner's burden to set forth a *prima facie* case. If the Examiner intends to rely on asserted evidence of what may or may not be true of one cancer as having relevance to the cancer type recited by the claims under examination, it becomes the Examiner's burden to show that there is a nexus between the two cancer types. The Examiner has not met this burden.

The Action states that the peptide of MAGE-A3 produced a very low level of CTL response which was detectable only by a sensitive method, such as that of Chaux. For the sake of good order, Applicants note that the method of Chaux was developed specifically to measure the level of anti-MAGE CTL precursors in individuals without cancer. See, Chaux at, e.g., title and at page 538, top right column. While the sensitive method of Chaux may be necessary to detect CTLs in persons without cancer, there is no apparent relevance to whether such methods would be necessary to detect such a response in persons with cancer.

Reconsideration, and withdrawal, of this portion of the rejection is respectfully requested.

vi.) Arguments that Bowie, Burgess, Lazar, and Scott show that it would be unpredictable that variants of the protein and peptides would function

The Action next argues that, given the alleged unpredictability of antigen expression of Boon, of induction of a strong immunogenic effect of Kirkin, and the teachings of Bowie, Burgess, Lazar and Scott that a change in a single amino acid regarding the effect of changing even a single amino acid, even if specific peptides of the invention stimulated T cell responses, it could not be predicted that the claimed variants would also function to stimulate T cell responses. According to the Action, it would therefore take undue experimentation to practice the invention. Action, at page 17. Close examination of the references, however, shows that they do not support the Action's conclusion.

Applicants note first that the argument that undue experimentation would be necessary to practice the invention relies on combining the premises that Boon establishes unpredictability of antigen expression and that Kirkin teaches that it is unpredictable that a strong immunogenic effect can be achieved with the alleged teachings of the Bowie, Burgess, Lazar and Scott references. As set forth in the preceding section, however, Applicants could not locate the passage of Boon allegedly making this point and Kirkin has not been shown to be relevant to the claims under examination. Accordingly, the first part of the Action's argument to be combined with the teachings of the other references does not appear to be present. Second, turning to the alleged teachings of the Bowie, Burgess, Lazar and Scott references, it has also been shown above that they are not apposite to the claims under examination. For the Examiner's convenience, a brief recap may be in order.

Bowie, Burgess, Lazar and Scott are applied to suggest that the change in a single amino acid might destroy the ability of the claimed peptides to be recognized by T cells from patients with mesothelin-expressing cancers. As noted previously, above, the references do not regard matters analogous to the present invention. These references relate to the biological

activity of the protein; none of the references relate to T cell recognition of a peptide or of a protein, and therefore lend no support to the Action's contentions. Further, the specific teachings of the references either support the claims under examination, or are not relevant to them.

As previously noted, Bowie teaches "that proteins are surprisingly tolerant of amino acid substitutions . . . For example, . . . about one-half of all substitutions were phenotypically silent [citation omitted] At some positions, many different, nonconservative substitutions were allowed." Bowie, at page 1306, right column, first full paragraph. Thus, Bowie would lead persons of skill to predict that many substitutions could be made in fragments of SEQ ID NO:2 without affecting the ability of T-cells activated by endogenous mesothelin in a patient with a mesothelin-expressing cancer to recognize the resulting peptides.

Lazar relates to the mutation of two specific amino acids known to be conserved in the same family of peptides as the peptide, TGF-α, that is the subject of the reference. The particular mutations made were calculated to change the characteristics of the amino acid at the conserved position, such as mutating an aspartic acid (which would normally be negatively charged at physiological pH) at position 47 of the molecule to an alanine or an asparagine (which would normally be uncharged at physiological pH). See, abstract. Not surprisingly, perhaps, these non-conservative mutations of a conserved residue changed the biological activity. There is no apparent evidence in the reference, however, that this mutated protein would not have been recognized by T cells that recognized the original TGF-α.

Burgess concerns the mutation of a residue known to be important to the biological activity of a particular growth factor (HBGF-1). Like Lazar, the mutation was a non-conservative mutation (from positively charged lysine to negatively charged glutamic acid), specifically because the lysine in question had been previously implicated as important to the biological activities of the growth factor. See, abstract. Not surprisingly, the activity was affected. What is not clear is what relevance this finding has to the recognition of the growth factor by a T cell.

Scott adds nothing to the Action's contention, since it merely concerns the ability to predict the ion transported by the product of the Pendred syndrome gene by its homology to

other transporters. (Applicants note in passing, however, that while the substrate may have been unpredictable, the transporter did still act as an ion transporter, as its homology suggested.) Since it does not concern mutating a sequence, it appears to be even less relevant than Lazar and Burgess to the claims under examination.

Accordingly, close examination shows that the references cited by the Action do not support the position that there is unpredictability in the claimed proteins and peptides. Thus, there is no basis to support the Action's conclusion that undue experimentation would be required to practice the invention as claimed. Reconsideration and withdrawal of the rejection are respectfully requested.

vii. Arguments drawn to rejection of canceled claim 17

The Action states that the arguments made by the Applicants with respect to now-canceled claim 17 are relevant to the instant rejection. In this regard, the Action asserts that it has considered Applicants' argument that there is no requirement that a vaccine cure a disease to be enabled. The Action states that the argument is not persuasive for the reasons set forth in the Action regarding the unpredictability of peptide vaccines. Action, at page 17. Further, the Action alleges that the Applicants' arguments to the effect that the claims are not drawn to replacement of standard therapies, that the Spitler reference is based on whole cell vaccines not relevant to the vaccines at issue, and that Evans et al. show that the immune system can be activated to attack cancers have been considered but not found persuasive due to the alleged unpredictability shown by the references discussed above.

As an initial matter, Applicants reiterate that the claims under examination recite compositions; they do not recite vaccines. Thus, Applicants respectfully maintain that rejecting the claims as now presented on grounds that vaccines are not enabled inappropriately imports into the claims a recitation that is not present, and rejects them for this imported recitation. The rejection as a whole is therefore procedurally flawed and should be reconsidered on this basis alone.

The rejection is also substantively flawed. The Action's argument that the peptide vaccines are unpredictable rests on combining two sets of references that, as shown in the preceding section, are not analogous to, or relevant to, the claims under examination. The argument therefore fails on this basis alone. Applicants also respectfully observe that the Action's argument also ignores the loading of peptides onto dendritic cells, a technique that was known prior to the priority date. The Action makes no allegation, let alone a showing, that there is any unpredictability for this use.

Accordingly, Applicants maintain that the Action fails to establish that there is any lack of enablement of the claims as presented. Reconsideration and withdrawal of the rejection is respectfully requested.

C. Rejection of claims 20-26 and 30-32 Under 35 U.S.C. § 112, first paragraph for lack of written description

The Action next rejects claims 20-26 and 30-32 under §112, first paragraph as assertedly lacking written description. The Action asserts that the specification does not contain the limitation of "at least 90% sequence identity" but rather refers to a 90% identity over a comparison window of 10-20 amino acids. The Action argues that in the absence of that limitation, the subject matter claimed in claims 20-26 and 30-32 broadens the scope of the invention as originally disclosed. Action, at pages 18-19. Applicants amend in part and traverse.

The Applicants maintain that the recitations of the claims as presented are supported by the specification. To expedite prosecution, however, claims 20 and 23 have been amended to recite that the proteins have 85% sequence identity to SEQ ID NO:2. Applicants respectfully note that page 6, lines 19-23, state that a polypeptide has substantial identity to another when it has 85% sequence identity to a reference polypeptide. Claims 21 and 30 have been amended to recite that the peptides have 90% sequence identity over a comparison window of about 10-20 amino acid residues to SEQ ID NO:2. The recitation tracks that of the specification at page 6, lines 23-25. The remaining claims are dependent on the claims amended herein. Accordingly, it is believed that the amendments obviate the rejection.

D. Rejection of claim 22 Under 35 U.S.C. § 112, first paragraph for lack of written description

The Action next rejects claim 22 as lacking written description. According to the Action, the recitation of "10 or more contiguous peptides" has no clear support. Applicants note this is a typographical error and have amended the claim to recite amino acids, rather than peptides.

E. Rejection of Claims 19-25 and 27-32 Under 35 U.S.C. § 112, first paragraph for lack of written description

The Action next rejects claims 19-25 and 27-32 as not supported by written description in the specification. The Action cites *University of California v. Eli Lilly and Co.*, 119 F.3d 1559 (Fed. Cir. 1997) and *Enzo Biochem Inc. v. Gen-Probe Inc.* as applying to the rejection. According to the Action, *Lilly* stated that the written description requirement requires a precise description such as by formula or chemical name, and that a genus requires recitation of a representative number of cDNAs or a recitation of structural features. Further, the Action states that *Enzo* clarified that the written description standard could be met by disclosure of sufficiently detailed relevant identifying characteristics. The Action concedes that *Lilly* and *Enzo* both involved DNA constructs, but states "the holdings of those cases are also applicable to claims such as those at issue here." Action at page 21. The Action then states that the present claims fail to meet the standards articulated in *Lilly* and *Enzo*. Action, at pages 21-25. Applicants traverse.

As noted, the Action concedes that the *Lilly* and *Enzo* cases speak to cDNA constructs, but asserts that "the holdings of those cases are also applicable to claims such as those at issue here" - that is, to claims to proteins and peptides, which are, of course, not DNA or cDNA molecules. Markedly absent from this assertion, however, is citation to any authority supporting the application of the holdings of *Lilly* and *Enzo*, which were developed in the context of DNA, to claims involving proteins and peptides. Further absent from the Action is any

reasoning explaining why this application should be made. It is respectfully maintained that application of the holdings of these cases to the present claims is inappropriate.

The courts have recognized that the degeneracy of the genetic code makes a large genus of DNA able to encode a single amino acid sequence, and therefore makes it impossible to predict from an amino acid sequence the natural DNA sequence that encodes it. MPEP §2163, *citing In re Bell*, 991 F.2d 781 (Fed. Cir. 1993). Thus, it is perhaps not surprising that the courts found in the cases cited that recitation of structural characteristics or a number of species was required to provide description of a genus of nucleic acids encoding a protein.

In contrast, the amino acids constituting the sequence of a polypeptide are not degenerate. The Office has recognized this difference by routinely allowing claims to peptides and proteins so long as they have a specified percent identity to a reference peptide or protein, and so long as there is a functional recitation.

In the instant case, the claims recite proteins and peptides have a defined degree of sequence identity to SEQ ID NO:2 or peptides thereof and which retain defined functions. Such claims are routinely accepted by the Office as complying with the statutory requisites for patenting, including the written description requirement, and the Action has presented no reason why the same treatment is not appropriate in this case. Reconsideration and withdrawal of the rejection are respectfully requested.

F. Rejection of Claim 19 Under 35 U.S.C. § 112, second paragraph as indefinite

The Action rejects claim 19 as indefinite for reciting "a" full length amino acid sequence of SEQ ID NO:2. The Action maintains that it is unclear whether the claim refers to the full length of SEQ ID NO:2 or to a full length of a fragment of SEQ ID NO:2.

Applicants respectfully maintain that claim 19 as presented cannot be read as suggested by the Action. The claim as presented did not read "the full length of a fragment" of SEQ ID NO:2, but of SEQ ID NO:2. The "a" was deemed required simply because there was no

antecedent for the use of the article "the." To expedite prosecution, however, the claim has been amended to refer to "the" full length of SEQ ID NO:2.

G. Rejection of Claim 32 Under 35 U.S.C. § 112, second paragraph as indefinite

Claim 32 was rejected as indefinite as lacking an antecedent for the phrase "mesothelin-derived" peptide. The claim has been amended to delete the phrase. Applicant respectfully submits that the amendment obviates the rejection.

H. Rejection of Claims 19-25 and 27-32 Under 35 U.S.C. § 102(b) as anticipated

The Action rejects claims 19-25 and 27-32 under §102(b) as anticipated by Chang et al., Cancer Research, 52:181-186 (1992) ("Chang"). According to the Action, it is not possible to determine whether SEQ ID NO:2 refers to the 69 kD precursor protein or to the processed 40 kD protein. The Action further asserts that the sequence of the 40 kD processed protein is unknown and therefore it will be assumed that it comprises at least 90% of SEQ ID NO:2. According to the Action, Chang "specifically teaches an isolated 40 kDa CAK1 protein (see Fig. 3, p. 184, and Discussion, col. 2, p. 184)." Applicants traverse.

The Action mistakes the band in Figure 3 of the Chang paper for showing the presence of an isolated 40 kDa CAK1 protein. This is not correct. Figure 3 is a photograph of a Western blot in which supernatant from cells digested treated with phosphatidylinositol-phospholipase C ("PI-PLC", see Materials and Methods, page 181, right hand column, under "Phosphatidylinositol-Phospholipase C Digestion") was separated by SDS-PAGE. The proteins on the gel were then transferred to a nitrocellulose paper and probed with the K1 antibody (Materials and Methods, page 182, left column, under "Western Blotting for PI-PLC Supernatant of HeLa and OVCAR-3 Cells"). The K1 antibody was able to show the presence of the CAK1 antigen in the supernatant.

What Figure 3 does not show, however, is the presence of the CAK1 antigen in isolation. A Western blot is designed precisely to reveal the protein bound by the probing antibody (if, of course, that protein is present), without also revealing the presence of any number of other proteins that may also be present, but which are not bound by the antibody used to probe the blot. Many membrane proteins are linked to the cell surface by phosphoinositol linkages and are released by PI-PLC treatment. ("Mesothelin is one of many proteins and glycoproteins that are attached to the cell surface by phosphatidylinositol." Chang and Pastan, Proc Natl Acad Sci USA 93:136-140 (January 9, 1996), at page 140, left column. A copy of this reference is enclosed.) Thus, while Figure 3 shows the presence the CAK1 antigen, it does not and was not intended to show the presence of the other many other membrane proteins and glycoproteins that were released by the PI-PLC digestion of the OVCAR-3 cells, and that were present in the supernatant probed in the Western blot. Accordingly, neither Figure 3 nor the statement in the Discussion that the authors had succeeded in detecting the antigen as a band on the Western blot show the presence of an isolated protein.

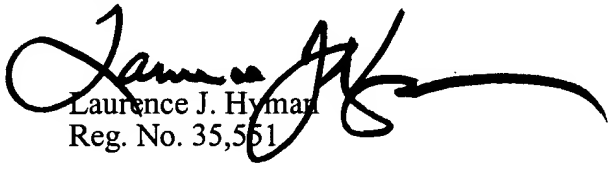
Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, she is requested to call the undersigned at 415-576-0200.

Respectfully submitted,



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Enclosures

60348254 v1

Strategies for the development of vaccines to treat breast cancer

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II. Medizinische Klinik, Hamatologie-Onkologie, Krankenhaus Nordwest, Frankfurt am Main, Germany.

The characterization of tumor-associated antigens recognized by cellular or humoral effectors of the immune system has opened new perspectives for cancer therapy. Several categories of cancer-associated antigens have been described as targets for cytotoxic T lymphocytes (CTLs) in vitro and in vivo: "cancer-testis" (CT) antigens expressed in different tumors and normal testis, melanocyte differentiation antigens, point mutations of normal genes, antigen that are overexpressed in malignant tissues, and viral antigens. Clinical studie using peptides derived from these antigens have been initiated to induce specific CTL responses in vivo. Immunological and clinical parameters for th assessment of peptide-specific reactions have been defined, i.e., induction of delayed-type hypersensitivity (DTH), CTL, autoimmune, and tumor regressio responses. Preliminary results demonstrate that tumor-associated peptides alo elicit specific DTH and CTL responses leading to tumor regression after intradermal injection. GM-CSF was proved to be effective in enhancing peptide-specific immune reactions by amplification of dermal peptide-presenting dendritic cells. Long-lasting complete tumor regressions have bee observed after induction of CTLs by peptide immunization. However, in a fe cases where there was disease progression after initial tumor response, loss o either the tumor antigen targeted by CTLs or of the presenting MHC class I molecule was detected as the mechanism of immune escape under immunization in vivo. Based on these observations, cytokines to enhance antigen and MHC class I expression in vivo are being evaluated to prevent immunoselection. Recently, a strategy utilizing spontaneous antibody respon to tumor-associated antigens (SEREX) has led to the identification of a new antigen, NY-ESO-1. In a melanoma patient with high titer antibody against N ESO-1, strong HLA-A2-restricted CTL reactivity against the same antigen w also found. Clinical studies involving tumor antigens that induce both antibo and CTL responses will show whether these are better candidates for immunotherapy of cancer.

Publication Types:
- Review

1: Cancer Biother Radiopharm. 1998 Jun;13(3):165-76.

Clinical experience with autologous tumor cell lines for patient-specific vaccine therapy in metastatic melanoma.

Dillman RO, Nayak SK, Barth NM, DeLeon C, Schwartzberg LS, Spitle Church C, O'Connor AA, Beutel LD.

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Because of their patient specificity and proliferative capacity, tumor cell line established from autologous metastatic melanoma tumor samples may be an excellent immunogen for patient-specific vaccine therapy. Between October and July 1996, the Hoag Cancer Center cell biology laboratory received 136 metastatic melanoma samples from 122 different patients. Tumor cell lines w successfully established for 92 of 136 samples (68%), for 87 of 122 patients Successful cultures were expanded to 10(8) cells (total culture time about 8 v confirmed to be sterile, irradiated, and stored frozen in aliquots of 10(7) cells Vaccines were prepared from 72 lines, and 62 vaccines were used in 57 diffe patients. Subcutaneous vaccination took place on weeks 1, 2 and 3, and then monthly for a total of 6 months. A delayed tumor hypersensitivity skin test (I was administered at week zero and week 4. Various adjuvants were co-administered including BCG, alpha- or gamma-interferon, and GM-CSF. Pat were monitored for failure-free survival (FFS) and overall survival (OS) fron date of the first vaccination. Follow-up data is available for 52 patients, 27 w no evident disease (NED) at the time of vaccination and 25 who had metasta disease at the time of treatment. There were two partial responses which pers 11.9 and 39.8+ months among the 25 patients who had detectable metastatic disease whun treatment was initiated (8%, 1 to 26%, 95%-Ci). Twenty patier negative skin tests at week 0 and week 4; six were positive both times, and 1 converted their DTH from negative to positive, for a conversion rate of 13 of (39%). Patients who received interferon-gamma and/or GM-CSF as an adjuv had a higher rate of DTH conversion compared to patients who received othe adjuvants (13 of 20 v 2 of 13, P = 0.003). For patients who were NED, nine c (47%) converted their DTH test compared to four of 14 (29%) patients with metastatic disease (p = 0.33). For patients whose DTH converted from negati positive after 3 weeks of vaccination, median FFS and OS were superior con to patients whose DTH remained negative (19.4 v 4.0 months FFS, p = 0.005 39.6 v 18.3 months OS, p = 0.0602). The autologous cell line approach to act specific immunotherapy is feasible for patients who have resectable foci of

metastatic disease. Administration of such patient-specific vaccines improves survival for those patients who are NED at the time of vaccination and conve DTH skin test, compared to those whose DTH test remains negative.

Publication Types:

- Clinical Trial
- Multicenter Study

PMID: 10850352 [PubMed - indexed for MEDLINE]

1: Nat Med. 1995 Dec;1(12):1297-302. Related Article

Bone marrow-derived dendritic cells pulsed with synthetic tumo peptides elicit protective and therapeutic antitumour immunity.

Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, CJ, Ildstad ST, Kast WM, Deleo AB, et al.

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Dendritic cells, the most potent 'professional' antigen-presenting cells, hold p for improving the immunotherapy of cancer. In three different well-character tumour models, naive mice injected with bone marrow-derived dendritic cell prepulsed with tumour-associated peptides previously characterized as being recognized by class I major histocompatibility complex-restricted cytotoxic T lymphocytes, developed a specific T-lymphocyte response and were protecte against a subsequent lethal tumour challenge. Moreover, in the C3 sarcoma a 3LL lung carcinoma murine models, treatment of animals bearing establishe macroscopic tumours (up to 1 cm2 in size) with tumour peptide-pulsed dendr cells resulted in sustained tumour regression and tumour-free status in more t 80% of cases. These results support the clinical use of tumour peptide-pulsed dendritic cells as components in developing effective cancer vaccines and therapies.

PMID: 7489412 [PubMed - indexed for MEDLINE]

1: J Exp Med. 1996 Jan 1;183(1):317-22.

Comment in:
- J Exp Med. 1996 Jan 1;183(1):7-11.

Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo.

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The priming of an immune response against a major histocompatibility comp class I-restricted antigen expressed by nonhematopoietic cells involves the tr of that antigen to a host bone marrow-derived antigen presenting cell (APC) presentation to CD8+ T lymphocytes. Dendritic cells (DC), as bone marrow-derived APC, are first candidates for presentation of tumor-associated antige (TAA). The aim of this study was to see whether DC are able to prime in viv antigen-specific cytotoxic T lymphocytes after exposure to a soluble protein antigen in vitro. Lacking a well-defined murine TAA, we took advantage of l galactosidase (beta-gal)-transduced tumor cell lines as a model in which beta operationally functions as TAA. For in vivo priming both a DC line, transduc not transduced with the gene coding for murine GM-CSF, and fresh bone ma derived DC (bm-DC), loaded in vitro with soluble beta-gal, were used. Primi with either granulocyte macrophage colony-stimulating factor-transduced DC or fresh bm-DC but not with untransduced DC line generated CTL able to ly beta-gal-transfected target cells. Furthermore, GM-CSF was necessary for th line to efficiently present soluble beta-gal as an H-2Ld-restricted peptide to a gal-specific CTL clone. Data also show that a long-lasting immunity against challenge can be induced using beta-gal-pulsed bm-DC as vaccine. These res indicate that effector cells can be recruited and activated in vivo by antigen-p DC, providing an efficient immune reaction against tumors.

PMID: 8551239 [PubMed - indexed for MEDLINE]

1: J Immunol. 1996 Apr 15;156(8):2918-26.

Induction of antitumor immunity using bone marrow-generated dendritic cells.

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We have previously shown that bone marrow-generated dendritic cells (DC) with a class I-restricted peptide are potent inducers of CD8+ CTL. In the pre study we have investigated whether bone marrow-generated DC are capable inducing antitumor immunity. We show that a single immunization with DC with OVA peptide was highly effective in eliciting a protective immune resp against a challenge with tumor cells expressing the OVA gene (E.G7-OVA), so than immunization with irradiated E.G7-OVA cells, OVA peptide-pulsed S cells, or free OVA peptide mixed with adjuvant. The addition of free OVA protein to day 4 or day 7 bone marrow cultures, but not to day 9 mature DC, also effective in eliciting CTL and engendering antitumor immunity, but was effective than peptide-pulsed DC. Induction of CTL and antitumor immunity bone marrow-generated DC pulsed with the class I-restricted OVA peptide correlated with the expression of syngeneic MHC class II molecules on the D This and the fact that induction of tumor immunity was dependent on CD4+ suggest that in vivo priming of CTL and induction of antitumor immunity by marrow-generated DC also require the presentation of MHC class II-restricte epitopes and activation of CD4+ T cells. This observation has potentially im implications to the use of peptide-pulsed DC in clinical immunotherapy.

PMID: 8609412 [PubMed - indexed for MEDLINE]

1: Eur J Immunol. 1995 Nov;25(11):3100-7. Related Article

Dendritic cells generated from peripheral blood transfected with human tyrosinase induce specific T cell activation.

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Peptides of melanosomal proteins have recently been shown to be recognized HLA-restricted mode by specific cytolytic T lymphocytes in melanoma patie Dendritic antigen-presenting cells (DC) are considered to be the most effecti stimulators of T cell responses, and the use of these cells has therefore been proposed to generate therapeutic responses to tumor antigens in cancer patier We, therefore, generated DC from peripheral blood of normal donors in the presence of granulocyte/macrophage colony-stimulating factor and interleuki Flow cytometric analysis of the cells during a 2-week culture revealed a loss CD14 and CD34 expression, a concomittent increase of CD1a, CD11a,b and CD44, CD45, CD54, HLA-class I and II, and intermediate levels of CD26, C and CD86. Cultured DC stimulated proliferation of allogeneic T cells and inc a marked, up to 20-fold, stimulation of T cell proliferation after pulsing with tetanus toxoid. To achieve independence of already-identified antigenic pepti presented in HLA class I-restricted fashion, which limits the general applicat of such peptides for vaccination of melanoma patients, we tested whether DC transfectable with eukaryotic expression plasmids. DC transfected with two reporter genes (CAT, beta-galactosidase) using a liposome-based transfectior technique, exhibited only low levels of enzymatically active proteins, but we to degrade rapidly intracellular proteins and to process peptides efficiently. Chloramphenicol acetyltransferase as well as tyrosinase mRNA were detecta after transfection by reverse-transcriptase-polymerase chain reaction, and enz activities became measurable. Furthermore, DC transfected with the tyrosina gene were able to induce specific T cell activation in vitro, indicating approp peptide processing and presentation in DC after transfection. These data sugg new approaches to future tumor vaccination strategies.

PMID: 7489749 [PubMed - indexed for MEDLINE]

1: Cancer Res. 1995 Nov 15;55(22):5330-4. Related Articles, L

Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antig derived epitopes by dendritic cells in vitro.

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MHC class I-restricted CTLs specific for antigens expressed by malignant ce are an important component of immune responses against human cancer. Recently, in melanoma a number of melanocyte differentiation antigens have been identified as potential tumor rejection antigens. In the present study, we show that by applying peptide-loaded dendritic cells, induced by granulocyte macrophage colony-stimulating factor and interleukin 4 from peripheral bloo monocytes of healthy donors, we were able to elicit melanoma-associated antigen-specific CTL in vitro. We demonstrate the induction of CTLs directe against HLA-A2.1 presented epitopes derived from tyrosinase, gp100, and Melan A/MART-1. Apart from lysis of peptide-loaded target cells, these CTl displayed reactivity with HLA-A2.1+ melanoma tumor cell lines and culture normal melanocytes endogenously expressing the target antigen. These data indicate that these CTLs recognize naturally processed and presented epitope and that precursor CTLs against melanocyte differentiation antigens are pres in healthy individuals. The ability to generate tumor-specific CTLs in vitro, using granulocyte-macrophage colony-stimulating factor/interleukin 4-induc dendritic cells, illustrates the potential use of this type of antigen-presenting cells for vaccination protocols in human cancer.

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Cell Biology

Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers

(glycosylphosphatidylinositol anchor/plasma membrane/antibody/cell adhesion)

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Contributed by Ira Pastan, October 9, 1995

ABSTRACT **Monoclonal antibody MAb K1 recognizes a 40-kDa glycoprotein present on the surface of mesothelial cells, mesotheliomas, and ovarian cancers. We have used MAb K1 to isolate a 2138-bp cDNA that encodes this antigen. The cDNA has an 1884-bp open reading frame encoding a 69-kDa protein. When the cDNA was transfected into COS and NIH 3T3 cells, the antigen was found on the cell surface and could be released by treatment with phosphatidylinositol-specific phospholipase C. The 69-kDa precursor is processed to the 40-kDa form. The protein has been named mesothelin because it is made by mesothelial cells. Mesothelin may play a role in cellular adhesion.**

Monoclonal antibodies are currently being used to diagnose and treat cancer (1, 2). To be useful for therapy, the antibody should recognize an antigen that is present in large amounts on the cancer cells and in negligible amounts on normal cells. Alternatively, the antigen can be present in substantial amounts on normal cells, if the normal cells are not components of an essential organ. This approach has been useful in developing new treatments for leukemias and lymphomas. Several differentiation antigens have been identified on lymphomas and leukemias which are good targets for immunotherapy because they are not present on the stem cells which give rise to differentiated lymphocytes (2). Thus, normal lymphocytes that are killed by immunotherapy can be regenerated. Some examples of lymphocyte antigens of this type are CD19, CD22, CD25, and CD30 (2, 3). Clearly, it would be very useful to have antibodies that recognize differentiation antigens on solid tumors, but only a small number of these are available. One reason contributing to the paucity of such antibodies is that efforts to identify differentiation antigens on various types of epithelial cells have been relatively modest compared with the intense efforts made to identify differentiation antigens on cells of the hematopoietic system.

Ovarian cancer represents one of the diseases which could be treated by immunotherapy, because the ovaries are always removed during surgery for this disease and reactivity with normal ovarian tissue is not a problem. Several antibodies that recognize differentiation antigens on ovarian cancer cells have been generated. One of these is OC125, which recognizes the CA125 antigen (4). CA125 is a high molecular weight glycoprotein that is shed by ovarian cancer cells and has been useful in the diagnosis of ovarian cancer. However, antibodies to CA125 are not useful for immunotherapy because the CA125 antigen is shed into the bloodstream (4). Another is MOV18, which recognizes the folate-binding protein. This protein is abundant in ovarian cancers as well as in some other tumors. Unfortunately, this protein is also abundantly expressed in kidney (5). We have isolated an antibody termed MAb K1 that reacts with many ovarian cancers and many mesotheliomas. Like OC125, the antibody also reacts with normal mesothelial cells, but it does not react with other cell types except for weak reactivity with some cells in the trachea (6, 7). The antigen recognized by MAb K1 appears to be a differentiation antigen present on mesothelium and is expressed on cancers derived from mesothelium, such as epithelioid type mesotheliomas, as well as on most ovarian cancers. Thus immunotherapy directed at the CAK1 antigen will run the risk of damaging normal mesothelial cells and perhaps cells of the trachea (6–9).

In the ovarian cancer cell line OVCAR-3 as well as HeLa cells, the antigen has been shown to be an ≈40-kDa glycoprotein that is attached to the cell surface by phosphatidylinositol. The protein is released when cells are treated with phosphatidylinositol-specific phospholipase C (PI-PLC) (7). We have previously attempted to clone a cDNA encoding the CAK1 antigen but instead have cloned cDNAs encoding two intracellular proteins which also react with MAb K1 (10). Neither of these is the cell surface antigen recognized by MAb K1. Here we describe the cloning of a cDNA that encodes the CAK1 antigen that is expressed on the cell surface.* We have expressed the cDNA in 3T3 cells and characterized the protein made by these cells.

MATERIALS AND METHODS

Cells and Antibodies. Human ovarian tumor cell line OVCAR-3 and cell lines A431, KB, MCF-7, COS-1, WI-38, and NIH 3T3 were obtained from the American Type Culture Collection. Cells were cultured either in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with L-glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml), and 5–10% fetal bovine serum (FBS) (GIBCO). NIH 3T3 transfectants were grown in DMEM with the neomycin analogue G418 (GIBCO) at 0.8 mg/ml. Cells were used when they reached 80–90% confluency, after three washes with ice-cold phosphate-buffered saline (PBS) (GIBCO). MAb K1 and antibody MOPC-21 have been described (6) and were used at 5–10 μg/ml.

Isolation of the cDNA Clones. The HeLa S3 cDNA library (Clonetech) was screened as described (10) at ≈50,000 plaque-forming units per 150-mm filter with protein A-purified MAb K1 (5 μg/ml) and peroxidase-conjugated goat anti-mouse IgG (heavy- and light-chain specific, 10 μg/ml; Jackson ImmunoResearch). Two positive plaques (λ6-1 and λ6-2) were isolated and the phages were purified to homogeneity by three or more rounds of screening. After verification of their specificity with MAb K1 by showing they did not react with a control MOPC-21 antibody, single-plaque isolates of λ6-1 and λ6-2 were used for preparation of 5–10 phage plates, followed by extraction and purification of phage DNA with a λ phage

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Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U40434).

DNA kit (Qiagen, Chatsworth, CA). Phage DNA was then digested with *Eco*RI and the insert was subcloned into the *Eco*RI site of a pcDNAI/Amp (Invitrogen) vector by a rapid ligation protocol (10). Plasmid DNAs were isolated by use of Qiagen's plasmid DNA isolation kit (10). Restriction mapping using *Xho* I, *Eco*RI, *Sal* I, *Bam*HI, and *Nco* I, as well as DNA sequencing, revealed that the two plasmid clones (p6-1 and p6-2) had identical 1500-bp inserts.

To isolate a longer clone, the insert of p6-1 was purified to make a cDNA probe (specific activity, 8.5×10^5 cpm/ml) by random priming. The HeLa S3 cDNA library was rescreened by a filter hybridization method (10). Fourteen λ clones were isolated and purified, and their insert sizes were assessed by digestion with *Eco*RI. Four large inserts were subcloned into a pcDNAI/Amp plasmid vector (p9, p13-1, p16, and p18-1). p9 contained the largest insert with a long open reading frame.

DNA Sequence Analysis. By the use of T3 and T7 promoter primers and twenty 17-bp synthetic primers, the entire cDNA insert of p9 was sequenced by the method described by Sanger *et al.* (11) and an automatic cycle sequencing method (Taq DyeDeoxy Terminator Cycle sequencing kit; Applied Biosystems).

Northern Blot Analysis. Total RNAs (20 μg) from OVCAR-3, KB, MCF-7, A431, and WI38 cells were electrophoresed in a 1% agarose gel in Mops buffer with 16.6% formaldehyde and then transferred to a nylon paper. Northern hybridization was done with a method described before (10). The blot was washed and reprobed with a ^{32}P-labeled human β-actin cDNA as an internal control to assess the integrity and quantity of the RNA samples loaded.

***In Vitro* Transcription and Translation.** The TNT coupled reticulocyte lysate system, canine pancreatic microsomal membrane, 2 μg of plasmid DNAs of pcDICAK1-9, pAPK1 (10), and [^{3}H]leucine were used in an *in vitro* transcription/translation and translocation/processing experiment according to the protocol of the manufacturer (Promega). Translation products were resolved by SDS/10% PAGE under reducing conditions. The proteins were fixed and the unincorporated label was removed by soaking the gel three times for 30 min in 200 ml of buffer containing 40% methanol and 10% acetic acid in deionized water. The gels were then soaked for 30 min in 200 ml of ENTENSIFY Part A and Part B (NEN). After drying, the translated products were visualized by autoradiography.

Expression of the Cloned cDNAs in Mammalian Cells. Transient transfections of COS cells were performed with pcDICAK1-9 (p9) and LipofectAMINE (GIBCO) according to the manufacturer's protocol. COS-1 cells were plated a day before the experiment at 2.5×10^5 cells per 60-mm dish. Twenty-four microliters of LipofectAMINE and 76 μl of OptiMEM I medium were mixed with 10 μg of pcDNAI/Amp vector or pcDICAK1-9 in 100 μl of OptiMEM-I medium at room temperature for 30 min. After the COS-1 cells were washed twice with OptiMEM-I 2.4 ml of OptiMEMI was added and the transfection mixtures were overlaid onto COS-1 cells and incubated at 37°C. After 5 hr, 2.6 ml of DMEM with 20% FBS was then added into each dish. Forty-eight hours after transfection, the dishes were subjected to immunofluorescence labeling (6, 7) or other treatments. The insert from pcDICAK1-9 (in pcDNAI/Amp) was also subcloned into a pcDNA3 (Invitrogen) vector for stable transfection. Plasmid minipreps were made with Qiagen's Miniprep plasmid DNA kit and orientation of the insert in individual clone was determined by restriction mapping. The resulting plasmid, pcD3CAK1-9, was then used to transfect NIH 3T3, MCF-7, A431, and OVCAR-3 cells by DNA–calcium phosphate precipitation (12). After overnight exposure to the precipitate, the cells were washed with PBS three times and fed with fresh DMEM/10% FBS for 2–3 days. Geneticin (G418 sulfate; 0.8 mg/ml) was added and the cultures were maintained until colonies 2–3 mm in diameter were formed. Colonies were then transferred into wells of a 96-well plate and then into a 35-mm dish when they were 80% confluent. Transfected cells were screened by immunofluorescence (6, 7) and positive cells were further subcloned by limited dilution as described (6). One of the NIH 3T3 transfectant clones, NIH 3T3 K20, was chosen for further study. To localize the expression of CAK1, both cell surface and intracellular immunofluorescence labeling was also performed according to methods described before (7).

Treatment of Transfected Cells with PI-PLC. CAK1 cDNA-transfected NIH 3T3 cells (NIH 3T3 K20 cells) were grown in 175-mm^2 flasks, and when they reached 90% confluency, the cells were washed in PBS three times. The cells were incubated with either 5 ml of PI-PLC (1.25 units/ml, from *Bacillus cereus*; Boehringer Mannheim) or 0.05% trypsin/0.052 mM EDTA for 30 min at 37°C and 30 min at room temperature with shaking. The supernatants were collected and after centrifugation at $1000 \times g$ and concentrated about 10-fold with a Centricon 30 unit (Amicon). The concentrated supernatants were used in SDS/PAGE and immunoblot analysis. The enzyme-treated cells can be recultured and the recovery of CAK1 expression can be seen after overnight culture. Treatment with PI-PLC was done in a similar manner using 35-mm diameter dishes followed by immunofluorescence labeling of the treated cells (7).

Immunoblot Analysis of Transfected NIH 3T3 Cells. Membrane and cytosolic fractions from transfected NIH 3T3 K20 cells (10) were subjected to SDS/12.5% PAGE and the resolved proteins were transferred to nitrocellulose. Immunoblotting was performed as described (8, 10).

RESULTS

Expression cloning was used to isolate the CAK1 cDNA. We previously observed that MAb K1 reacts with OVCAR-3 and HeLa cells. Because we were unable to isolate the cDNA from an OVCAR-3 library (10), we screened a HeLa cDNA library expressed in λgt11 as described in *Materials and Methods.* A total of 1×10^6 phages were screened and 2 phage clones (λ6-1 and λ6-2) were identified. DNA sequencing showed that both phages contained the same 1.5-kb insert. The insert hybridized to mRNA from OVCAR-3 and KB cells (a HeLa subclone which also reacts with MAb K1) but not to RNA from many other cell lines, indicating that the cDNA is specific for cells reacting with MAb K1 (Fig. 1). The mRNA detected was 2.2 kb long, indicating that the insert isolated was not full-length. The insert contained an open reading frame, a stop codon, and a poly(A) tail but the 5′ end appeared to be missing. Therefore, the phage library was rescreened with one of the inserts and 14

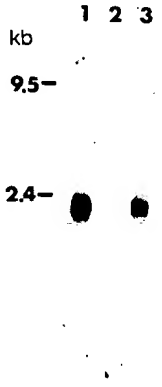

FIG. 1. CAK1 RNA levels in cell lines. Samples (20 μg) of total RNA from OVCAR-3 cells (lane 1), MCF-7 cells (lane 2), KB cells (a HeLa subclone; lane 3), A431 cells (lane 4), and W138 cells (lane 5) were resolved by electrophoresis, transferred to nylon paper, and probed with a ^{32}P-labeled CAK1 cDNA. Hybridization with an actin cDNA probe showed that the lanes were equally loaded.

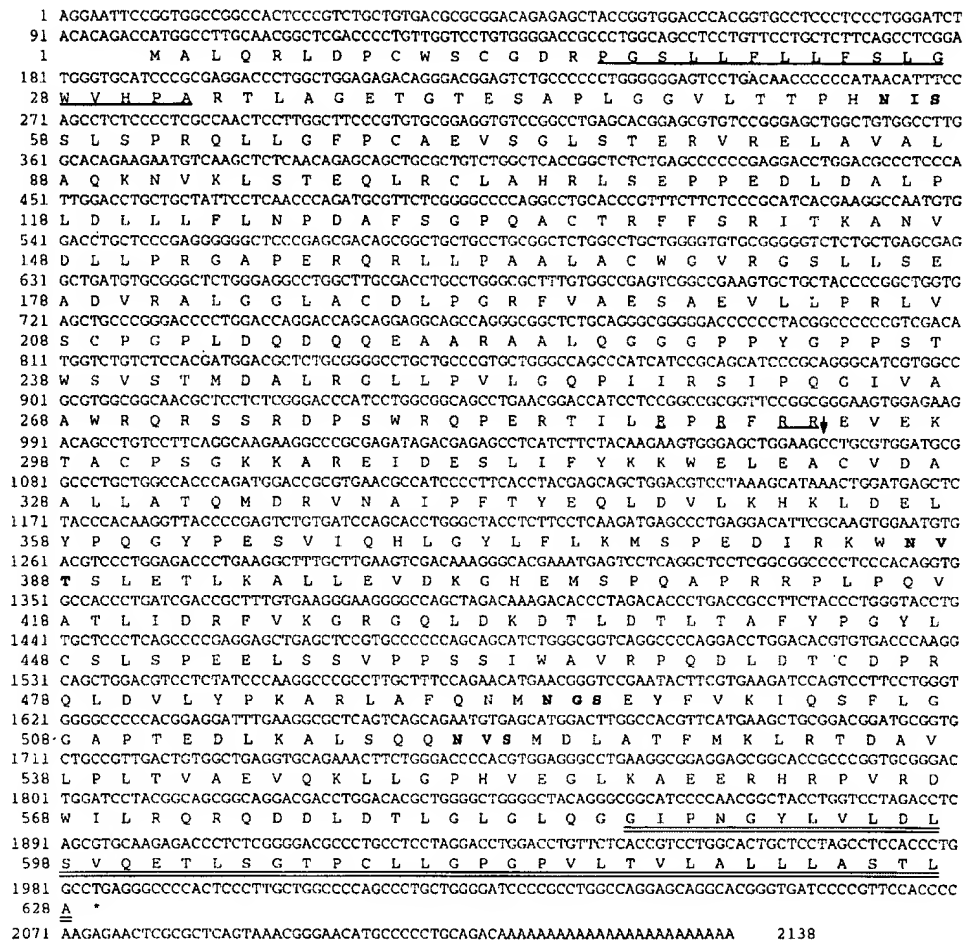

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the CAK1-9 cDNA. The nucleotide sequence (upper line) and the deduced amino acid sequence (lower line) of the CAK1-9 cDNA are listed. The translation of CAK1-9 starts at nucleotides 100–102 (ATG) and terminates at 1986–1988 (TGA). The putative signal peptide is underlined and a typical hydrophobic sequence for glycosylphosphatidylinositol anchorage is doubly underlined. A possible furin cleavage sequence, RPRFRR, is underlined and the cleavage site is shown by an arrow. There are four potential N-linked glycosylation sites (in boldface letters). A variant polyadenylylation signal (AGTAAA) is present 22 bp upstream from the poly(A) tail. The original p6-1 cDNA sequence spans nucleotides 721–2138.

new phages with cDNA inserts of various sizes were isolated. The largest insert (no. 9) was 2138 bp long and when sequenced contained an open reading frame of 1884 bp (Fig. 2). It contains a typical Kozak sequence (13) (AXXATGG) followed by an open reading frame that encodes a 69 kDa protein. The sequence was not present in various data bases examined (EMBL/GenBank release 89.0, June 1995). Because the CAK1 antigen was originally found to be about 40 kDa in size, several experiments were carried out to determine whether clone 9 encoded CAK1.

***In Vitro* Translation.** Insert 9 was cloned into a pcDNAI/Amp vector to make pcDICAK1-9 and used in the TNT reticulocyte system. A 69-kDa protein was produced (Fig. 3, lane 1). In the presence of pancreatic microsomes a slightly larger protein was observed (lane 2), indicating that the protein had been inserted into microsomes and glycosylated. As a control, a cDNA encoding a 30-kDa cytosolic protein that also reacts with MAb K1 (10) was subjected to the same analysis. The size of that protein was unaffected by the presence of microsomes (lanes 3 and 4).

Expression in Cultured Cells. pcDICAK1-9 was transfected into COS cells for transient expression. Fig. 4 shows the specific MAb K1 labeling pattern of COS cells transfected with insert 9. In nonpermeabilized cells, a typical cell surface fluorescent pattern was detected (Fig. 4*A*). In permeabilized cells, strong staining of the Golgi region was evident (Fig. 4*B*). No cytosolic staining was detected. Also, no immunoreactivity was detected in cells transfected with vector without insert (Fig. 4*C*) or with control inserts (data not shown). Thus, insert 9 encodes a cell surface protein that is also present in the Golgi compartment.

Size and Processing of CAK1 Antigen. To determine the size of the antigen produced by cells transfected with insert 9, NIH 3T3 cells were transfected with pcD3CAK1-9 to make stable cell lines. Stably transfected clones were produced as described in *Materials and Methods* and the presence of antigen on the surface was confirmed by immunofluorescence. Then mem-

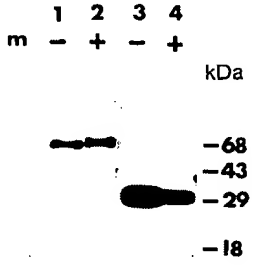

FIG. 3. *In vitro* translation of CAK1 cDNA. Plasmids pcDICAK1-9 (lanes 1 and 2) and pcDIAPK1 (lanes 3 and 4) were used in a TNT coupled reticulocyte lysate system in the presence (+) or absence (–) of pancreatic microsomal membrane (m). The products were resolved by SDS/10% PAGE under reducing conditions and autoradiographed.

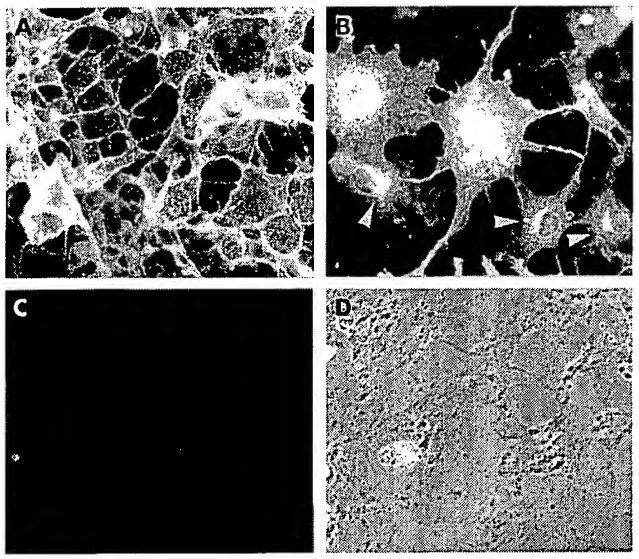

FIG. 4. Immunofluorescence of CAK1-9-transfected cells. pcDNAI/Amp vectors with insert 9 (*A* and *B*) or without insert (*C* and *D*) were transfected into COS cells. Two days later, the cells were immunocytochemically labeled with antibody MAb K1 at 4°C (for surface labeling, *A*) or at 23°C (for intracellular labeling, *B*) and photographed. Arrows indicate the typical Golgi distribution of the recombinant CAK1 in the transfected cells. (×145.)

brane and cytosolic fractions were prepared from NIH 3T3 K20 cells and from OVCAR-3 cells, subjected to SDS/PAGE, and analyzed by immunoblotting with MAb K1 (Fig. 5). As previously reported, the major reactivity in OVCAR-3 cells was with a doublet of about 40 and 43 kDa that was present in membranes but not in the cytosol. In the transfectants, two bands of equal intensity were detected in the membrane fraction; one of about 40 kDa and a second of about 71 kDa. No signal was detected in the cytosol. These data suggest that CAK1 is made as a high molecular weight precursor that is processed by proteolysis to an ≈40-kDa form.

Nature of Cell Surface Attachment. To determine whether CAK1 was attached to the transfectants via a phosphatidylinositol linkage as it is in OVCAR-3 cells (7), the NIH 3T3 transfectant cell line K20 was treated with PI-PLC for 60 min. Fig. 6*A* shows the strong cell surface labeling pattern in untreated cells. Fig. 6*C* shows that fluorescence was absent after treatment with PI-PLC. Fig. 6 *B* and *D* show phase-contrast images before and after treatment, respectively. The treated cells were still attached to the dish but were slightly altered in shape. The medium from PI-PLC-treated cells was concentrated, subjected to SDS/PAGE, and analyzed with MAb K1. A band of about 70 kDa was detected (data not shown), but no lower molecular weight bands were detected.

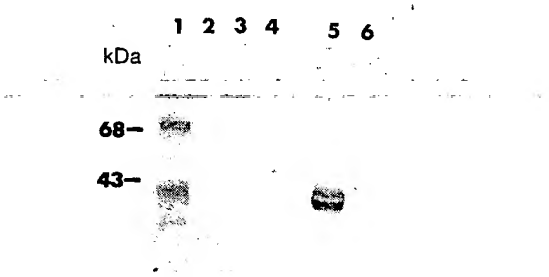

FIG. 5. Immunoblot analysis of OVCAR-3 cells and NIH 3T3 cells transfected with CAK1-9 cDNA. Approximately 100 μg of membrane fraction (lanes 1 and 3) or cytosolic fraction (lanes 2 and 4) of the transfected NIH 3T3 (pcD3CAK1) and mock control (pcD3) and membrane (lane 5) or cytosolic fraction (lane 6) of OVCAR-3 cells were electrophoresed and immunoblotted with MAb K1.

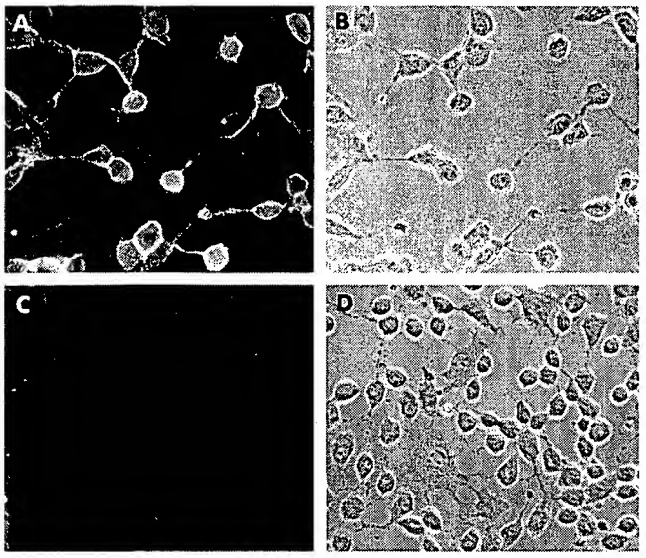

FIG. 6. Removal of the recombinant CAK1 by PI-PLC treatment of the transfected NIH 3T3 cells as assessed by immunofluorescence. The transfected NIH 3T3 K20 cells were treated with PI-PLC and labeled with MAb K1 as described in *Materials and Methods*. *A* and *B* show NIH 3T3 K20 cells without PI-PLC treatment. *C* and *D* demonstrate that the CAK1 signal was completely abolished after PI-PLC treatment. *B* and *D* are phase-contrast images. (×145.)

DISCUSSION

This paper describes the molecular cloning of the CAK1 antigen, which is found on mesothelium, mesotheliomas, ovarian cancers, and some squamous cell carcinomas. We have named this antigen mesothelin to reflect its presence on mesothelial cells. One unexpected feature of mesothelin is that its cDNA encodes a 69-kDa protein, whereas the antigen present on OVCAR-3 cells, used to isolate MAb K1, has a molecular mass of ≈40 kDa. The DNA sequence and the deduced amino acid sequence of CAK1 are shown in Fig. 2. The cDNA is 2138 bp long and contains an open reading frame of 1884 bp. The protein it encodes contains 628 amino acids with a calculated molecular mass of 69,001 Da. A homology analysis was performed with nucleotide or amino acid sequences and none was detected with EMBL/GenBank accessed by the Genetics Computer Group program. The protein contains four potential N-linked glycosylation sites, NXS or NXT, that are shown in boldface letters. A typical signal sequence is not present at the amino terminus. However, a short hydrophobic segment is located 15 amino acids from the first methionine (Fig. 2). This sequence might function as a signal sequence for membrane insertion, because the protein is found on the cell surface (Figs. 4 and 5) and is inserted into microsomes during cell-free translation (Fig. 3). Also present is a putative proteolytic processing site, RPRFRR, beginning at amino acid 293 (Fig. 2). This site is recognized by furin, a protease important in the processing of several membrane proteins as well as in the activation of *Pseudomonas* and diphtheria toxins (14). The 40-kDa form appears to be derived from a 69-kDa precursor by several processing steps. These are summarized in Fig. 7. Initially, mesothelin is made as a 69-kDa polypeptide with a hydrophobic tail which is probably removed and replaced by phosphatidylinositol (7). After glycosylation at one or more of its four putative N-linked glycosylation sites, it is cleaved by a protease to yield the 40-kDa fragment (or doublet) found on the surface of OVCAR-3 cells and a smaller (≈31-kDa) fragment. The latter could be released into the medium and/or further degraded. The amino-terminal fragment has recently been detected in the medium of OVCAR-3 cells (unpublished data). In transfected NIH 3T3 and MCF-7

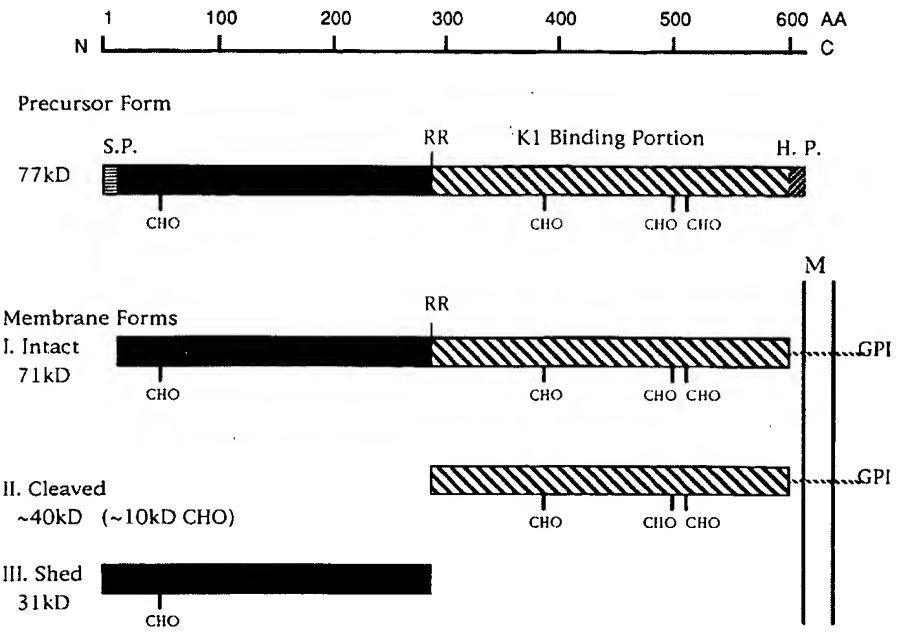

FIG. 7. Different forms of the CAK1 tumor antigen. S.P., putative signal peptide; H.P., glycosylphosphatidylinositol (GPI)-anchorage-dependent hydrophobic peptide; CHO, carbohydrates; M, membrane; AA, amino acids.

cells, we find approximately equal amounts of the 70-kDa and 40-kDa proteins. We originally detected the 40-kDa form in OVCAR-3 and HeLa cells and did not notice a larger form. Reexamination of the OVCAR-3 and HeLa cell gels reveals a trace amount of the 70-kDa precursor (7). We have not yet examined the size of mesothelin in human tissues or cancer samples or in other human cell lines to determine its size or the nature of its processing.

Mesothelin is one of many proteins and glycoproteins that are attached to the cell surface by phosphatidylinositol. Several functions have been ascribed to these molecules. Some are receptors involved in cell signaling; others are involved in cellular recognition and/or adhesion (15, 16). Glycosylphosphatidylinositol-linked proteins may interact with tyrosine kinases (17, 18). Currently we have very little information on the function of mesothelin. One possibility is that it has a role in adhesion, since CAK1 transfects are more slowly removed from culture dishes than nontransfected cells (unpublished data). Mesothelial cells are extremely flat and regulate the traffic of molecules and cells in and out of the peritoneal cavity. Mesothelin may have a role in these processes. Mesothelin could also be responsible for the adhesion and implantation of ovarian carcinoma cells that frequently occur throughout the peritoneal cavity. The isolation of a cDNA clone that encodes mesothelin will make investigation of these phenomena possible.

Mesothelin is very abundant in normal mesothelial cells from which malignant mesotheliomas and ovarian cystadenocarcinomas are derived. These two types of tumors share a unique biological characteristic that distinguishes them from other solid tumors. In the early stages, both types of tumors spread aggressively throughout the peritoneal (or thoracic) cavity and invade locally but do not metastasize distally through the lymphatic system or the bloodstream. In fact, many patients succumb to their cancer before distant metastases develop. Very little is known about the molecules involved in the dissemination of mesotheliomas and ovarian cancers. Mesothelin may have a role in this process, since cells overexpressing mesothelin have altered adhesive properties (unpublished data) and mesothelin expression is diminished in poorly differentiated ovarian cancers (8, 9). Investigation of mesothelin should not only advance our knowledge of mesothelial cell function but also promote our understanding of the role of this glycoprotein in carcinogenesis, invasion, and metastasis of ovarian cancers and malignant mesotheliomas.

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